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Energetics of Isolated Hepatocyte Swelling Induced by Sodium Co-transported Amino Acids

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This study was designed to investigate the energetics of isolated rat hepatocyte swelling due to sodium-cotransported amino acid accumulation in a medium containing either glucose or octanoate as basal substrate. We show that the size of the increase in cytosolic volume is directly correlated with the total amino acid accumulation, which depends on the difference of electrical potential across the plasma membrane. Such a change in cell volume, with either glucose or octanoate, does not modify the mitochondrial volume. Addition of sodium-cotransported amino acids for which the metabolism was avoided showed that the rise in cell volume, *per se*, did not change the respiratory rate, Δp, or phosphate potential in either mitochondrial or cytosolic compartments. Conversely, the large increase in oxidative phosphorylation flux was due to the metabolism of amino acids as a consequence of a rise in electron supply for the respiratory chain rather than an increase in cellular ATP demand, as indicated by the increase in cytosolic phosphate potential. Moreover, although we confirm that octanoate addition largely increases the respiratory rate by a process different from uncoupling, we observed that the same overall thermodynamic driving force through the respiratory chain and the same mitochondrial or cytosolic phosphate potential were maintained for much higher oxygen consumption when octanoate was present. We propose that these octanoate effects are due to a decrease in the actual protons/2 electrons stoichiometry as a consequence of a shift in electron supply toward a two-coupling site instead of a three-coupling site. The change in the $FADH_2$/NADH formation flux ratio in either fatty acid or carbohydrate oxidation explains such results.

KEY WORDS: Hepatocytes; cell swelling; oxidative phosphorylation; amino acids; octanoate.

INTRODUCTION

It is now well known that an increase in cell volume, both in perfused liver and isolated hepatocytes, affects fluxes through some metabolic pathways acting like an anabolic signal (see Haüssinger and Lang, 1991; Haüssinger *et al.*, 1994, and Hue, 1994 for reviews), stimulating and inhibiting the biosynthesis and degradation pathways, respectively. Cell volume increase can be a consequence of either a decrease in extracellular osmolarity or an incubation with Na^+-cotransported amino acids (Bacquet *et al.*, 1990). Thus, cell swelling induced by either hypoosmotic medium, amino acid exposure, or insulin (i) inhibits glycogenolysis, glycolysis (Graf *et al.*, 1988; Lang *et al.*, 1989), proteolysis (Haüssinger *et al.*, 1990a; Haüssinger and Lang, 1991; Haüssinger *et al.*, 1991; Hallbrucker *et al.*, 1991), glutamine synthesis and urea synthesis from NH_4^+ (Haüssinger *et al.*, 1990b; Haüssinger and Lang, 1990), and carnitine palmitoyltransferase I (Guzman *et al.*, 1994); (ii) stimulates glycogen synthesis (Bacquet *et al.*, 1990), protein synthesis (Haüssinger *et al.*, 1990c), amino acid uptake (Haüssinger *et al.*, 1990b,d; Haüssinger and Lang, 1990; Bode and Kilberg, 1991), glutaminase (Haüssinger *et al.*, 1990d) and urea synthesis from amino acids (Haüssinger *et al.*, 1990d; Haüssinger and Lang, 1990). On the other hand, hyperosmotic medium or glucagon-induced cell shrinkage (i) stimulates proteolysis, glycogenolysis, and glycolysis; (ii) inhibits protein synthesis, glycogen synthesis, and glutaminase activity.

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While the intermediary metabolism has been extensively studied and some mechanisms linking cell volume changes and the alterations of metabolic function has been proposed (Meijer *et al.*, 1992; Baquet *et al.*, 1993; Meijer *et al.*, 1993; Vökl *et al.*, 1994), little is known in this field about the energetic behavior during cell swelling or cell shrinkage. In a previous paper (Espié *et al.*, 1995), we showed that in isolated hepatocytes incubated in hypoosmotic media, cell swelling induces a large increase in mitochondrial volume which does not involve an activation of respiration. Moreover, measurements of the two forces controlling the respiratory rate, i.e., redox span through the respiratory chain and the protonmotive force, clearly indicate an increase in kinetic constraints over this metabolic pathway. In the literature, an increase in mitochondrial volume is often associated with an activation of respiration, either as a consequence of hormonal action *in situ*, or in hypoosmotic media *in vitro* (Armston *et al.*, 1982; Halestrap, 1989; Halestrap *et al.*, 1990). However, it should be noted that in their experiments performed with hepatocytes, the increase in respiration was always linked to an ion uptake in an unchanged external medium osmolarity. Thus, the respiration rate increase could be a consequence of a matrix ion accumulation rather than a rise in mitochondrial volume *per se*. In our experiments, it is clear that water movements are the main process involved in mitochondrial osmotic pressure adjustment inducing a decrease in matrix ion concentration. This can explain the apparent contradiction between these different results, and points to the necessity to investigate the cellular energetics and the mitochondrial behavior *in situ*, under each particular experimental condition.

While the response of the intermediate metabolism to cell swelling appears to be constant, one may expect that the putative modifications in energetics would depend on the way in which cells swell. Indeed, hepatocyte exposure to hypotonic medium induces a decrease in cytosolic osmolarity, thus inducing mitochondrial swelling without any change in oxidative phosphorylation rate (Espié *et al.*, 1995). In contrast, active accumulation of amino acids cotransported with sodium may produce an increase in both cytosolic osmolarity and respiratory substrates whose consequences on mitochondrial metabolism are unknown.

This study was designed to investigate the energetics of cell swelling due to sodium-cotransported amino acid accumulation. We show that the size of the increase in isolated hepatocyte volume is directly correlated to the total amino acid accumulation, which depends on the difference of electrical potential across the plasma membrane. Such changes in cell volume, with either glucose or octanoate as substrates, do not modify the mitochondrial volume. The rise in cell volume, *per se*, does not change respiratory rate, Δp,[2] or phosphate potential in either mitochondrial or cytosolic compartments. An increase in oxidative phosphorylation flux is due to the metabolism of amino acids and mainly to the rise in electron supply for the respiratory chain as indicated by an increase in cytosolic phosphate potential. Moreover, if we can confirm that octanoate addition does not uncouple oxidative phophorylation, we may propose that it induces a large stimulation in respiratory rate which is mainly due to a decrease in the actual protons/2 electrons stoichiometry as a consequence of the shift in electron supply toward a two-coupling site instead of a three-coupling site.

EXPERIMENTAL PROCEDURES

Preparation and Incubation of Hepatocytes

Hepatocytes from 20–24 h starved male Wistar rats (200–250 g) were isolated by the collagenase method of Berry and Friend (1969) as modified by Groen *et al.* (1982). Hepatocytes (8–10 mg dry weight/ml) were incubated in 20 ml stopped plastic or glass vials in a shaking water bath. The basic incubation medium was a Krebs–Henseleit–bicarbonate buffer pH 7.4 (Krebs and Henseleit, 1932) containing 2.5 mM Ca^{2+}, 2% defatted bovine serum albumin, either 20 mM glucose or 4 mM octanoate as indicated, 0.1 μM $TPMP^+$ (tetraphenylmethylphosphonium), 0.5 mM mannitol, and 0.2 mg/ml inulin. Amino acids (proline, alanine, glutamine, and 2-aminoisobutyrate) when added were used at 10 mM, except for leucine 2.5 mM. All media were in equilibrium with a gas phase containing O_2:CO_2 (95:5). The temperature was 37°C.

Measurement of Respiration Rate

After 25 min incubation, the cell suspension was transferred to an oxygen electrode for determination of respiration rate. Myxothiazol and oligomycin were

[2] Abbreviations: AIB: 2-aminoisobutyrate; AOA: aminooxyacetate; CCCP: carbonyl cyanide *m*-chlorophenylhydrazone; $\Delta G'_p$: Gibbs free-energy of ATP hydrolysis reaction; so-called phosphate potential; $\Delta E'_h$: Gibbs free-energy difference in oxidation reaction; Δp: protonmotive force; $\Delta\Psi_m$: mitochondrial transmembrane difference in electrical potential; $\Delta\Psi_p$: cellular transmembrane difference in electrical potential.

used at respectively 2 μg/ml and 25 μg/ml to determine the myxothiazol- and oligomycin-sensitive respiration rate. Carbonyl cyanide *m*-chlorophenylhydrazone and aminooxyacetate were used at 15 μM and 1 mM respectively.

Measurement of Cell and Mitochondria Volume *in situ*

Hepatocytes were incubated in the presence of (i) 2.5 μCi/ml ^3H_2O and 0.1 μCi/ml [^{14}C]carboxymethylinulin (added 1 min before sampling) for measurement of cell volume; (ii) 2.5 μCi/ml ^3H_2O and 0.1 μCi/ml [^{14}C]mannitol for measurement of mitochondrial volume (Quinlan *et al.*, 1983). Indeed, it is well known that water minus mannitol volume is an accurate measurement of the miochondrial matrix volume in accordance with electron microscopy estimation (see Halestrap, 1989 for review). After 25 min incubation, 1 ml of cell suspension was transferred to an Eppendorf tube and centrifuged for 5s at 7600 *g*. Within 10 s, 100 μl of the supernatant was removed into a scintillation vial containing 5 ml of a liquid scintillation cocktail (Beckman), and the remainder was aspirated. The pellet was resuspended with 300 μl $HClO_4$ 10%, centrifuged, and 200 μl of the supernatant treated as before. The volume of each isotope in the pellet was (dpm_c 3/2)/(dpm_e/100) in μl.

Measurement of Mitochondrial and Cytosolic Membrane Potential *in situ*

Despite the existence of some channels involved in Cl^- movement through the plasma membrane, it has been mainly proved that in hepatocytes, electrogenic Cl^- conductance is great enough to equilibrate Cl^- with plasma membrane $\Delta\Psi$ at least for a membrane potential below 40 mV (Nobes and Brand, 1989; Wang and Wondergem, 1991). Hepatocytes were incubated in the presence of (i) 1 μCi/ml [^{3}H]TPMP$^+$ for measurement of TPMP$^+$ accumulation; (ii) 0.1 μCi/ml $^{36}Cl^-$ for measurement of Cl^- distribution (Nobes and Brand, 1989). Cell samples were treated as above. The [^{3}H]TPMP$^+$ accumulation ratio and the $^{36}Cl^-$ distribution were calculated as in Nobes *et al.* (1990). Thus, the mitochondrial potential is

$$\Delta\Psi_m(i-e) = -60 \log \frac{V_c a_m}{V_m a_{cy}} \times \left(\frac{[TPMP^+]_i [Cl^-]_i}{[TPMP^+]_e [Cl^-]_e} \frac{(V_m + V_c) a_{cy}}{V_c a_e} - 1 \right)$$

(see Scott and Nicholls, 1980; Nobes *et al.*, 1990; Berry *et al.*, 1988; Hoek *et al.*, 1980). V_c, V_m, a_e, a_{cy}, a_m refer respectively to cellular and mitochondrial volumes, and apparent activity coefficients for TPMP$^+$ in the extracellular medium, the cytoplasm, and the mitochondrial matrix. We have previously redetermined a_e (because of our different extracellular medium), as well as a_{cy} and a_m (see Nobes *et al.*, 1990 and Murphy and Brand, 1987 for experimental procedures), and we take $a_e = 0.85$, $a_{cy} = 0.43$, $a_m = 0.38$ (Espié *et al.*, 1995).

Compartmentation of Nucleotides, Phosphate, and Amino Acids

The mitochondrial and cytosolic distribution of ATP, ADP, AMP, Pi and of the various amino acids assayed was studied by using the digitonin fractionation method as described by Zuurendonck and Tager (1974) (see also Akerboom *et al.*, 1979), with a slight modification concerning the volumes used. Concerning the amino acids, the amount of supernatants in the different pellets was determined using [^{14}C]inulin as the extracellular marker or [^{14}C]mannitol as the extramitochondrial marker, and the contaminating volume of the supernatant was subtracted.

Measurement of Intramitochondrial Redox Potential

The intramitochondrial NADH/NAD$^+$ ratio was determined by the metabolite indicator method (Akerboom *et al.*, 1979) assuming the β-hydroxybutyrate dehydrogenase reaction is in near-equilibrium (K_{app} = [AcAc] [NADH/βOHBu] [NAD$^+$], i.e., 4.93×10^{-2}; Krebs and Veech, 1969).

Assays

β-Hydroxybutyrate and acetoacetate were measured spectrophotometrically or fluorometrically in neutralized $HClO_4$ extracts (Bergmeyer, 1970); ATP, ADP, and AMP were measured by HPLC, using a reverse phase (Spherisorb, ODS II, 5 μm) column (0.46 × 25 cm) at 30°C. Elution was performed with a 25 mM sodium pyrophosphate/pyrophosphoric acid (pH 5.75) buffer at a flow rate of 1.2 ml/min. ATP, ADP, and AMP were eluted after 4.2, 5.0, and 8.4 min

respectively. The adenine nucleotide detection was performed at 254 nm, and the determination was linear in a range of 3–3,000 pmol. Pi was measured according to the method of Berenblum and Chain (1938). The amino acids (in neutralized $HClO_4$ extracts) were derivated in phenylthiocarbamyl-amino acids and separated by HPLC at 254 nm (Henrikson and Meredith, 1984).

RESULTS

Volumes and Amino Acid Distribution

It has previously been shown that hepatocytes incubated with glucose swell when different sodium cotransported amino acids are added (Bacquet *et al.*, 1990). Similar results were obtained with octanoate (Table I) or oleate (not shown) instead of glucose. In the control conditions or in the presence of amino acids (except for glutamine + leucine), cellular volumes with octanoate were slightly greater than those observed with glucose. Even if AIB (a nonmetabolizable but Na^+-cotransported amino acid; Shotwell *et al.*, 1983) induced only a weak increase in cellular volume, the fact that added amino acid was not metabolized did not inhibit cellular swelling as shown by comparison between alanine alone and alanine + AOA, a potent inhibitor of transaminases (Cornell *et al.*, 1984).

It is generally accepted that the use of [^{14}C]mannitol as an extramitochondrial space marker is the appropriate method to determine the matrix volume *in situ* (Halestrap, 1989). In this way, under control conditions on glucose and octanoate, we obtained values of 0.36 and 0.35 μl per mg cell protein respectively, which are identical to those of this author. Whatever the conditions and the cell volumes, the mitochondrial matrix volume remained unchanged (Table I). These observations led us to study the compartmentation of amino acids in hepatocytes. Indeed, when hepatocytes are incubated with sodium cotransported amino acids under steady state, their active accumulation must induce a rise in cytosolic osmolarity (Christensen, 1990). Figure 1 shows that there is a linear relationship between cytosolic volume and amino acid concentration. However, such a relationship seems to be different with either glucose or octanoate; for the same amino acid concentration, the cytosolic volume is always slightly higher with octanoate.

Kristensen and Folke (1984) reported high steady-state intracellular alanine concentrations when 10 mM alanine was added to isolated hepatocytes whose alanine metabolism was inhibited by AOA. Cell swelling was accompanied by increase in membrane K^+ permeability (Cohen and Lechene, 1990) and hyperpolarization of cell transmembrane potential (Fitz and Scharschmidt, 1987; Wondergerm and Castillo, 1988).

Table I. Effects of Amino Acid Additions on Cellular and Mitochondrial Volumes[a]

Additions	V_c μl · mg^{-1} cell proteins	V_m μl · mg^{-1} cell proteins
Glucose, control	2.75 ± 0.15 (10)	0.36 ± 0.04 (5)
proline	3.27 ± 0.18 (5)	0.32 ± 0.04 (5)
alanine	3.36 ± 0.12 (5)	0.37 ± 0.05 (5)
glutamine	3.11 ± 0.11 (4)	0.32 ± 0.04 (4)
glutamine + leucine	3.47 ± 0.09 (6)	0.33 ± 0.05 (4)
AIB	2.89 ± 0.05 (3)	0.33 ± 0.05 (3)
alanine + AOA	3.44 ± 0.15 (2)	0.38 ± 0.05 (2)
Octanoate, control	2.89 ± 0.14 (12)	0.35 ± 0.03 (8)
proline	3.66 ± 0.20 (6)	0.37 ± 0.03 (4)
alanine	3.58 ± 0.14 (5)	0.34 ± 0.06 (4)
glutamine	3.32 ± 0.18 (5)	0.33 ± 0.04 (4)
glutamine + leucine	3.37 ± 0.19 (6)	0.35 ± 0.05 (4)
AIB	3.06 ± 0.13 (4)	0.33 ± 0.05 (3)
alanine + AOA	3.67 ± 0.20 (3)	0.33 ± 0.07 (3)

[a] Hepatocytes were incubated and cellular (V_c) and mitochondrial (V_m) volumes were determined as described in Experimental Procedures. The incubation medium contained either 20 mM glucose or 4 mM octanoate and, as indicated, different amino acids (10 mM except for leucine 2.5 mM). Aminooxyacetate (AOA) was at 1 mM when present. Values reported are means ± S.D. for (n) cellular preparations.

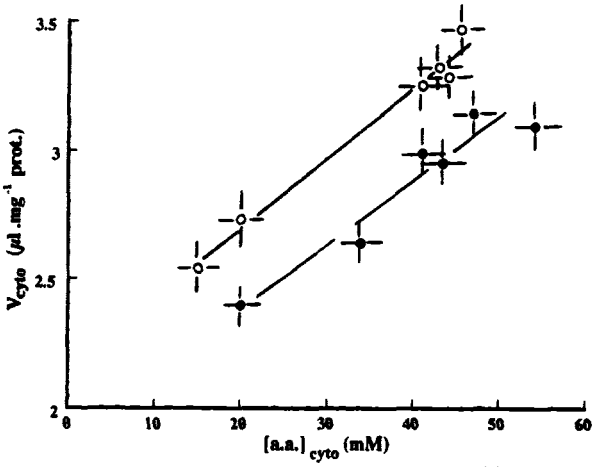

Fig. 1. Relationships between volume and amino acid concentration in cytosol of isolated hepatocytes. Hepatocytes were incubated as described in Experimental Procedures in the presence of either 20 mM glucose (●) or 4 mM octanoate (○). Cytosolic volume was estimated as the difference between cellular and mitochondrial volumes. When total cytosolic amino acid concentration was increased the experimental conditions were: (i) with glucose, control, AIB, glutamine, proline, alanine, and glutamine + leucine; (ii) with octanoate, control, AIB, glutamine, glutamine+leucine, alanine, and proline. The external added amino acid concentration was 10 mM, except for leucine (2.5 mM), and was not significantly modified during the time of incubation used. Data present means ± S.E.M. from at least three independent determinations, with individual experiments performed in duplicate.

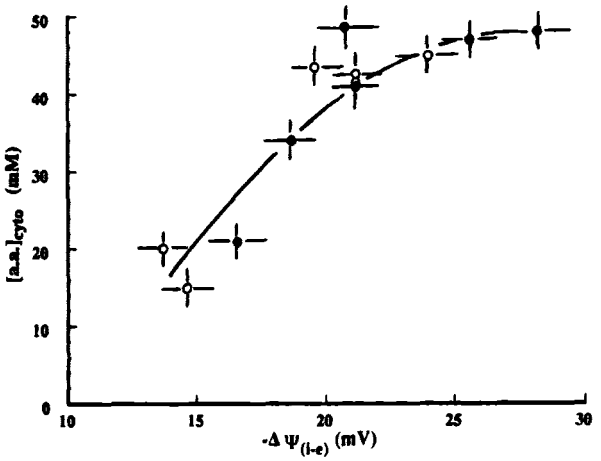

Fig. 2. Dependence of cytosolic amino acid concentration on the difference of electrical potential across the plasma membrane. $\Delta\psi$ was determined from the Cl^- distribution as described in Experimental Procedures. When $-\Delta\Psi$ increased, the experimental conditions were: (i) with glucose (●), control, AIB, glutamine, alanine, glutamine + leucine, and proline; (ii) with octanoate (○), AIB, control, proline, alanine, glutamine, and glutamine + leucine. Data present means ± S.E.M. from at least three independent determinations, with individual experiments performed in duplicate.

In fact, whatever the amino acid added, in steady state, the transmembrane potential (out–in) largely increased (Fig. 2). From a thermodynamic point of view, the amino acid distribution is dependent on the sodium electrochemical potential difference and not only on plasma membrane electrical potential difference. Meanwhile, there is a unique relationship between the cytosolic amino acid concentration and the value of the transmembrane potential: with either glucose or octanoate, when the transmembrane potential increased from 14 mV to 22 mV, the amino acid concentration rose from 15 mM to about 50 mM. This seems to indicate that sodium distribution is not significantly modified under our incubation conditions. Higher hyperpolarization to 28 mV was observed without a significant increase in amino acid concentration. From the analysis of the amino acid content of cytosol and mitochondria, we can calculate a concentration ratio between both these compartments (mitochondrial versus cytosolic) under the different experimental conditions (Fig. 3). This ratio was about 3 in control experiments with octanoate. It largely decreased after amino acid additions from about 1.5 with either alanine, glutamine, or glutamine plus leucine to 1.1 and 1 with AIB and proline respectively. Thus, a two- or threefold decrease in the mitochondrial versus cytosolic ratio of amino acids in hepatocytes incubated with octanoate did not significantly modify the mitochondrial volume *in situ* (see Table I). With glucose, in control experiments, the amino acid concentration ratio was equal to two (Fig. 3B). Its decrease after amino acid addition was low (below 25%) except for AIB: in this case, we observed an 80% drop without any mitochondrial volume change.

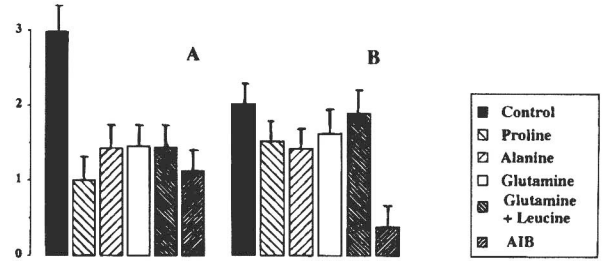

Fig. 3. Mitochondrial versus cytosolic amino acid concentration ratio in isolated hepatocytes. Hepatocytes were incubated as described in Experimental Procedures with either 20 mM glucose (A) or 4 mM octanoate (B). Data present means ± S.E.M. from at least three independent determinations, with individual experiments performed in duplicate.

Respiratory Rate and Energetic Parameters

Respiratory rate was estimated as the oxygen consumption sensitive to myxothiazol at a concentration that completely inhibited the electron transfer through the respiratory chain. The myxothiazol-insensitive oxygen consumption was 7.5 ± 1.8 (n = 4) and 5.7 ± 1.6 natom O · min^{-1} · mg $protein^{-1}$ (n = 5) on glucose and octanoate respectively. Amino acid additions did not change these values (not shown). As previously reported (Nobes *et al.*, 1990), addition of fatty acid raised the respiratory rate by about 90% and the mitochondrial membrane electrical potential difference from 152 to 160 mV (Table II). The addition of metabolizable amino acids increased the respiratory rate on both glucose and octanoate to a similar extent (between 7 and 10 natom O · min^{-1} · mg $protein^{-1}$) and, in both cases, slightly decreased the mitochondrial $\Delta\Psi$. However, a nonmetabolizable amino acid (AIB) did not significantly change the respiratory rate or $\Delta\Psi$. Moreover, alanine, in the presence of aminooxyacetate which prevents its metabolism, induced only a slight rise in respiratory rate on glucose without any effect on $\Delta\Psi$ and had no effect on octanoate (Table II). Thus, amino acid metabolism is responsible for both the increase in respiratory rate and the slight decrease in $\Delta\Psi$. This could be the consequence of an activation of some ATP-consuming processes by a precursor supply. However, the maximal respiratory rate obtained by uncoupler addition (CCCP) was always higher when metabolizable amino acids were present (Table II). This increase was very high on octanoate with both proline and glutamine. The fact that uncoupled respiration was enhanced by amino acid additions clearly indicates their actual participation in the electron supply pathway. There is no simple relationship between respiratory rate and either $\Delta\Psi_m$ (see Table II) or the mitochondrial NADH/NAD^+ ratio (not shown). However, from a thermodynamic point of view, the respiratory rate is controlled by two associated forces: the span of the redox potential over the respiratory chain ($\Delta E'_h$) and the protonmotive force (Δp). As there is no unquestionable method to assess quantitatively ΔpH across the inner mitochondrial membrane on isolated hepatocytes and as the $\Delta\Psi$ is the main component of the Δp, we express this overall thermodynamic driving force over the electron transport chain as $2\Delta E'h - n\Delta\Psi$, where n is the H^+/O stoichiometry of the electron transport chain. For substrates giving their electrons to complex I, it is generally assumed that n is equal to 10 (Stoner, 1987). But, it is worth noting that the absolute value of this stoichiometry does not play any

Table II. Effects of Different Amino Acid Additions on Mitochondrial Difference of Electrical Potential and Coupled and Uncoupled Respiratory Activity[a]

Additions		$\Delta\Psi$ mito (mV)	JO_2 (natom · min^{-1} · mg^{-1} protein)	
			−CCCP	+CCCP
Glucose, control	(10)	152 ± 4	19.8 ± 1.4	30.9 ± 2.7
proline	(5)	142 ± 4	29.8 ± 1.8	36.3 ± 3.0
alanine	(6)	142 ± 5	28.5 ± 3.0	33.2 ± 1.7
glutamine	(6)	148 ± 4	27.2 ± 2.2	43.3 ± 3.0
glutamine + leucine	(5)	144 ± 3	29.6 ± 2.0	42.7 ± 2.7
AIB	(3)	153 ± 4	18.9 ± 1.7	25.6 ± 2.4
alanine + AOA	(3)	151 ± 4	23.8 ± 1.3	32.2 ± 1.7
Octanoate, control	(9)	160 ± 4	36.8 ± 1.2	39.4 ± 2.7
proline	(6)	157 ± 7	43.5 ± 2.9	75.7 ± 3.3
alanine	(5)	152 ± 6	43.8 ± 1.9	56.6 ± 3.0
glutamine	(5)	156 ± 4	42.8 ± 3.7	88.3 ± 3.6
glutamine + leucine	(6)	150 ± 5	46.0 ± 3.9	88.3 ± 4.0
AIB	(3)	166 ± 5	36.6 ± 3.0	39.6 ± 2.2
alanine + AOA	(3)	162 ± 6	35.6 ± 1.7	40.9 ± 3.0

[a] Hepatocytes were incubated as described in Experimental Procedure in medium containing either 20 mM glucose or 4 mM octanoate and as indicated different amino acids (10 mM except for leucine 2.5 mM). Aminooxyacetate (AOA) was at 1 mM where present. $\Delta\Psi$ values were calculated as described in Experimental Procedure. Uncoupled respiratory rate was obtained after addition of 15 μM CCCP to reach the maximal oxygen consumption flux. Values reported were mean ± SD for (n) cellular preparations.

role in the shape of the relationship between respiratory rate and the overall thermodynamic driving force. From Fig. 4, it is clear that when the respiratory flux was manipulated by amino acid additions, two distinct and parallel relationships were observed, one in the presence of glucose and another with octanoate: for a similar overall thermodynamic driving force, the respiratory rate was much higher in the presence of octanoate. This difference between the two curves is independent of the chosen n value. With the assumption that cytosolic pH is constant, Nobes *et al.* (1990) have estimated that on isolated hepatocytes, mitochondrial ΔpH is only slightly lowered with octanoate as substrate instead of glucose. Even if we could not affirm that mitochondrial ΔpH is not affected under our different experimental conditions, slight variations in ΔpH under various conditions could not account for the displacement between the two curves in Fig. 4.

ATP, ADP, and Pi were measured in both cytosol and mitochondria under the different conditions described in Table I, except that with alanine in the presence of aminooxyacetate. Figure 5 shows that amino acid additions increase the Gibbs free energy of ATP hydrolysis with either glucose or octanoate in both mitochondrial and cytosolic compartments. Moreover, an identical mitochondrial or cytosolic ΔG_p was obtained for a higher respiration rate when octanoate was present as compared to glucose.

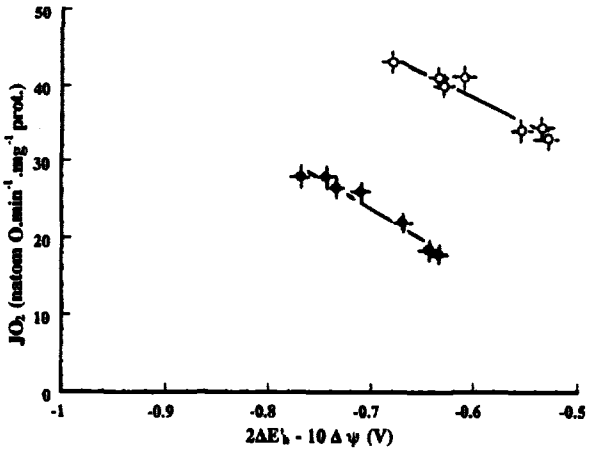

Fig. 4. Relationships between respiratory rate and the overall thermodynamic driving force. The basic incubation medium and the experimental conditions were as in previous figures, i.e., with glucose (●) and with octanoate (○). $\Delta E'_h$ is the difference in redox potential across the electron transport chain and n is the selected proton/2 electrons stoichiometry of the respiratory chain (n = 10, see text). Data present means ± S.E.M. from at least three independent determinations, with individual experiments performed in duplicate.

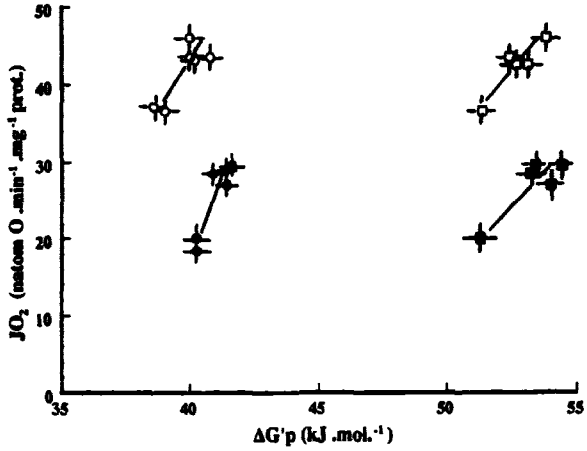

Fig. 5. Relationships between respiratory rate and either cytosolic or mitochondrial Gibbs free energy of ATP hydrolysis reaction. $\Delta G'_p$ values were calculated from the ATP, ADP, and Pi concentrations in both compartments (cytosol and mitochondria) using the digitonin fractionation method as indicated in Experimental Procedures. Mitochondrial $\Delta G'p$ with glucose (●) and with octanoate (○); Cytosolic $\Delta G'p$ with glucose (■) and with octanoate (□). Data present means ± S.E.M. from at least three independent determinations, with individual experiments performed in duplicate.

DISCUSSION

When hepatocytes are incubated with sodium-cotransported amino acids, their intensive accumulation induces a rise in cytosolic osmolarity (Kristensen, 1986). The sodium influx leads to a transitory depolarization of the plasma membrane, which is followed by a stable hyperpolarization due to an increase of potassium permeability (Fitz and Scharschmidt, 1987; Wondergerm and Castillo, 1988). Moreover, a correlation between this hyperpolarization and an increase in the initial rate of alanine transport has been shown (Moule *et al.*, 1987; Wang and Wondergem, 1993). In this study, we observed a unique relationship between the increase in the electrical potential difference across the plasma membrane (at values less than 25 mV) and the rise in the cytosolic steady-state concentration of amino acids, whatever the substrate (glucose or octanoate) or the amino acid concerned. This shows that in this range of values, the steady-state intracellular concentration of cotransported amino acids is essentially determined by the $\Delta\Psi p$ value. The increase in cytosolic osmolarity does not change the mitochondrial volume, indicating an osmotic pressure adjustment on both sides of the inner membrane. When glucose is present, this could be due to amino acid uptake since, at steady state, the mitochondrial versus cytosolic ratio only slightly decreases, except

for AIB which does not enter mitochondria. In the latter case and when amino acids are added in the presence of octanoate, the amino acid concentration ratio largely decreases and other osmotically active substances must be transported into the mitochondria. In a previous study, we showed that isolated rat liver mitochondria suspended in hyperosmotic KCl medium are able to regulate the matrix volume in such a way that under steady state, matrix volume recovery is complete (Devin *et al.*, 1997). This is due to a Pi-K salt entry dependent on Δp. Moreover, in the presence of external potassium, hyperosmolarity, under steady state, has no effect on energetic parameters such as respiratory rate, proton electrochemical potential difference, or oxidative phosphorylation efficiency (Devin *et al.*, 1997). This is in complete agreement with our observations when the amino acids accumulated are not metabolized. Indeed, under these conditions, the increase in cell volume and cytosolic amino acid concentration does not change mitochondrial volume, respiratory rate, $\Delta\Psi_m$, or phosphate potential. Thus, from an energetic point of view, the change in oxidative phosphorylation rate is strictly related to the metabolism of amino acids and not to a change in cell volume *per se*. Metabolism of amino acids can produce a substrate for both respiratory chain and different intermediary metabolisms which are known to be activated by cell swelling. The addition of metabolizable amino acids largely increases the uncoupled respiratory rate. Moreover, an increase in phosphorylating respiration due to amino acids is linked to an increase in phosphate potential in both mitochondrial and cytosolic compartments (see Fig. 5). Taken together, these facts indicate that amino acids activate the oxidative phosphorylation rate through the substrate supply rather than by increasing the ATP consumption. From a previous study (Gustafson *et al.*, 1994) performed under the same experimental conditions, it is possible to estimate the ATP cost of the stimulation of some metabolic pathways, i.e., urea and glycogen synthesis (**Table III**). If we take into account a theoretical ATP/O ratio of 2.6 (Hinkle *et al.*, 1991), in the presence of glucose, this energetic expenditure is about 20% (proline), 30% (alanine), and 50% (glutamine) of the total increase in ATP production. However, it is likely that the actual ATP/O ratio in hepatocytes is lower than this theoretical value and depends on both the nature of the real substrates consumed and the mitochondrial oxygen consumption rate. Brand *et al.* (1993) proposed an effective ATP/O value between 1.65 and 1.29. These values in the presence of added glucose appear rather low, and we have estimated the actual ATP/O under each of our experimental conditions. Considering the dependence between the effective ATP/O ratio and the respiratory rate presented in Devin *et al.* (1996) and the steady-state respiratory rate in isolated hepatocytes, we calculated an effective ATP/O of about 1.9 which is not significantly different under the four conditions presented in Table III. For this value, the energetic expenditure due to glycogen plus urea synthesis may represent about 28% with proline, 37% with alanine, and 69% with glutamine of the whole increase in ATP production. Thus, the part of the ATP turnover due to the activation of these metabolic pathways is dependent on the amino acid used and is very high with glutamine. Other cell functions may participate in the rise in ATP turnover such as protein synthesis and Na-K-ATPase, for instance. Moreover, it is worth noting that the change in phosphate potential itself must act on cellular ATP turnover.

The effects of fatty acids on cellular respiration have been known for a long time (for a review, see Wojtczak and Schönfeld, 1993). It has been reported by Nobes *et al.* (1990) that the increase in respiratory rate due to fatty acid addition in isolated hepatocytes is accompanied by a large increase in mitochondrial NADH/NAD^+ ratio which is associated with a low but significant increase in $\Delta\Psi_m$. These authors proposed that fatty acid addition to isolated hepatocytes raises the respiratory rate by increasing the mitochondrial NADH supply to the respiratory chain. Although membrane proton conductance is not change, the proton leak increases as a direct consequence of a rise in protonmotive force, thus contributing to the increase in respiratory rate together with an increase in Δp consuming processes. Even if some of our results presented here are similar and, as previously claimed by Nobes *et al.* (1990), not in agreement with an uncoupling effect of fatty acid, we show that the effects of octanoate are not attributable only to an increase in mitochondrial NADH supply. Indeed, by comparing the effects of amino acid additions in the presence of either glucose or octanoate, we observed that for the same overall thermodynamic driving force over the electron transport chain, the respiratory rate is much higher with octanoate (see Fig. 4). The shift in the relationship between the respiratory flux and the overall thermodynamic driving force when these parameters are manipulated by amino acid additions, in the presence of either glucose or octanoate, is independent of the value of the stoichiometry protons/2 electrons chosen. This may indicate a large activation

Table III. Energetic Expenditure Linked to Urea and Glycogen Synthesis in the Presence of Glucose and Amino Acids

Incubation conditions	JO_2 (natom O · min^{-1} · mg^{-1} protein)	J_{urea}[a]	$J_{glycogen}$[a]	$\Delta J_{ATP\ consumed}$ (calculated) (I)	$\Delta J_{ATP\ synthesized}$ (calculated) (II)	ΔJ (I)/ΔJ (II) (%)
		nmol · min^{-1} · mg^{-1} protein		nmol · min^{-1} · mg^{-1} protein		
Control	18.6	0.3	0.2	—	—	—
+ Proline	28	1.6	1.4	5.0	18	28
+ Alanine	26.8	2.1	0.7	5.8	15.6	37
+ Glutamine	25.6	3.0	1.1	9.1	13.3	69

[a] ref.: Gustafson *et al.* (1994) *Eur. J. Biochem.* **223**, 553–556.

$\Delta J_{ATP\ consumed}$ (I) was that necessary for extra urea and glycogen synthesis induced by addition of amino acids as indicated in the table.

$\Delta J_{ATP\ synthesized}$ (II) was the extra ATP synthesis linked to the increase in respiratory rate due to amino acid addition, considering an ATP/O equal to 1.9.

by octanoate of one or more enzymes of the respiratory chain itself leading to a change in the response of electron flux to its associated forces. Even if this hypothesis may not be discarded, considering the differences in the oxidative pathways of octanoate and glucose, another hypothesis is to be proposed. Since fatty acids are metabolized by β-oxidation, they lead to a higher proportion of $FADH_2$-reducing equivalents than substrates metabolized through glycolysis and the Krebs cycle. This shift in electron supply toward a two-coupling site system instead of a three-coupling site leads to a decrease in the overall protons/2 electrons apparent stoichiometry of the respiratory chain, which may be phenomenologically considered as equivalent to an intrinsic respiratory chain uncoupling. In such a case, the increase in respiratory rate is not accompanied by a decrease in $\Delta\Psi_m$ or phosphate potential as observed in this work. Thus, the difference in the relationship between respiratory rate and the overall thermodynamic driving force is certainly due to the change in the actual stoichiometry (n) of the respiratory chain. Hence, if we consider that the same rate of proton extrusion is obtained at the same overall thermodynamic driving force with either glucose or octanoate, this means that the actual stoichiometry is 8.4 and 10 with octanoate and glucose, respectively. These values are in agreement with the change in the formation flux ratio of $FADH_2$/NADH in fatty acid or carbohydrate oxidation.

In conclusion, in this work we show that the size of the increase in isolated hepatocyte volume is directly correlated with the amino acid accumulation, which depends on the difference of electrical potential across the plasma membrane. Such a change in cell volume does not modify the mitochondrial volume. An increase in oxidative phosphorylation flux is due to the metabolism of amino acids and mainly to the rise in electron supply for the respiratory chain. Moreover, although we confirm that octanoate addition does not uncouple oxidative phosphorylation, we propose that the large increase in respiratory rate is mainly due to a decrease in the actual protons/2 electrons stoichiometry as a consequence of the shift in electron supply toward a two-coupling site instead of a three-coupling site.

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